



ACCLIMATATION OF PINEAPPLE (*Ananas comosus* L.) PLANTS RESULTING FROM *IN VITRO* BUDDING AND SOMATIC EMBRYOGENESIS IN COTE D'IVOIRE

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ABSTRACT

Pineapple occupies an important place in the economy of Côte d'Ivoire. The quality of the planting material used in pineapple cultivation is a guarantee of improved profitability and fruit quality. To overcome this constraint, *in vitro* culture uses budding and somatic embryogenesis methods for a massive production of good sanitary quality plants. However, during acclimatization, some major difficulties due to the rot of plant's tissue culture and the nature of the substrate used disrupt production. Sea sand, dark soil layer and dark soil layer mixed with sawdust in a 1:1 (v/v) ratio were used as acclimatization substrates to evaluate the potentialities of Smooth Cayenne vitroplants. During acclimatization, under shade and in the field, survival rate, growth and development parameters of vitroplants were measured. Pineapple field-grown suckers were used as controls. The results showed that a 98% of the plantlets survived and the morphological uniform plants were observed on dark soil layer mixed with sawdust during weaning. The parameters observed, showed no significant differences between vitroplants. However, the vegetative growth parameters in the field and those related to yield were significantly ($p > 0.05$) different between the three types of plants. The Productivity of plants regenerated by somatic embryogenesis are more efficient than those regenerated by direct organogenesis. In addition, vitroplants have been more efficient than plants obtained traditionally. This difference in behaviour is due to the degree of rejuvenation of the plants by *in vitro* methods. The potential for massive production of quality plantlets by *in vitro* cultivation is therefore an asset for the renewal of the Ivorian orchard in view of the revaluation of pineapple from Côte d'Ivoire in the world. Acclimatization is a delicate and important step in *in vitro* culture. Its control will open up new possibilities for the farming world.

KEYWORDS: Vitroplants, substrate, weaning, breeding, field, behaviour.

INTRODUCTION

Pineapple [*Ananas comosus* var *comosus* (L.) Merrill Coppens and Leal] (Coppens and Leal, 2003), is native of South America. Pineapple is one of the most widely grown fruit crops in the tropics and subtropics. It is the eleventh most widely grown fruit (Kouadio, 2018). It holds the third rank in world tropical fruits only preceded by banana and citrus with a production of about 25.44 million tons (FAOSTAT, 2015). Pineapple culture occupies an important place in the economy of more than 80 countries. In Côte d'Ivoire, pineapple contributes to 0.6% of the national gross domestic product and 1.6 % of the agricultural gross domestic product. Indeed, the export of fresh pineapple generates nearly 30 billion dollars in revenue per year (CNE-CI, 2017). In Côte d'Ivoire, the variety Smooth Cayenne is the most cultivated because of its adaptation to soil, climate conditions, for its yield potential and its appreciation on the international market (Leal and Coppens d'Eeckenbrugge, 1996). The quasi-hegemony of smooth Cayenne cultivation in Côte d'Ivoire has resulted, after several decades of intensive cultivation, without fallow, a degeneration of the plant material with

enormous phytosanitary risks. This has led to an ageing of the Ivorian orchard with its consequent decrease in production and quality. Thus, there was a drastic drop in production, of about 90.4 %, in 2014 compared to 1999 from 213620 à 170 000 tonnes (CNE-CI, 2017).

Faced with these problems, the renewal of the ageing orchard seems essential. To overcome this constraint, *in vitro* regeneration seems to be an effective alternative to provide producers with plants of good sanitary qualities in order to revive pineapple cultivation in Côte d'Ivoire (Yapo, 2013). However, regenerated plants must undergo acclimatization, which is a critical phase of *in vitro* regeneration before being transferred on field. Indeed, the transition of vitroplants from *in vitro* conditions to very different natural conditions such as substrate structure and texture, availability of nutrients, humidity and temperature, aggressiveness of micro and macro organisms absent *in vitro etc.* represents a physiological stress for plants (Sidibé *et al.*, 2013). Studies have also shown that the type of acclimatization substrate can influence the *ex-vitro* development of vitroplants (Dossoukpevi *et al.*, 2015; Gnamien, 2016), thus affecting

the success and profitability of micropropagation. In addition, the survival rate of plantlets is generally low (Dibi, 2011). For the same cultivar, the agronomic performances of plants differ according to the initial propagation method (Youmbi *et al.*, 2005). Also, to obtain good pineapple fruit, it is advisable to favour the right size ones because they give more chance to produce at the end of the first year.

In order to provide performing material to producers, the general objective of this work is to study the influence of the substrate during acclimatization on the quality of the suckers and fruit production. The specific objectives are to evaluate the survival rate and parameters of vegetative growth and development of plantlets during acclimatization and in the field, and those related to vitroplant yield.

MATERIALS AND METHODS

Study site

The tests were conducted at the experimental station of Nangui Abrogoua University (Abidjan, Côte d'Ivoire). Located at 5°17' and 5°31' North latitude and 3°45' and 4°2' West longitude. The climate is humid tropical type. Temperatures and average precipitation based on data recorded on February 2014 were 26.2 °C and 1504.61 mm per year, respectively. The soil of the study site is ferruginous and loose type. The pH of this soil is more acidic on the surface (Coulibaly *et al.*, 2019).

Plant material

Plant materials were of rooted Smooth Cayenne vitroplants obtained by direct organogenesis and somatic embryogenesis according to the routine protocol established in the laboratory (Yapo *et al.*, 2011; Yapo, 2013). Traditional plants were used as controls in the field.

Methods

Acclimatization of vitro plants was carried out in two stages: weaning and breeding.

Weaning plants

Weaning consisting in a gradual adaptation of vitro plants to external conditions was carried out in two phases.

For the first phase, three different acclimatization substrates were tested. These are sea sand, arab land caught in fallow covered by vegetation and a mixture of arab land and sawdust in a 1:1 (v/v) ratio. These substrates were autoclaved at 121 °C for 30 min at a pressure of 1

bar. After cooling, the substrates were placed in plastic bins previously perforated at the base and then watered with distilled water. The following day, plantlets about 12 cm long with well-developed roots were removed from the agar culture media. The roots were thoroughly rinsed with tap water to remove any traces of agar. Then, 20 plantlets from each *vitro* method were transplanted by tray containing the different substrates (Fig. 1A). The bins were covered with a transparent plastic holder and kept in the culture room at 25°C under a 12 hours photoperiod for two weeks. Water was supplied to plants once kept in two days. A total of 120 vitroplants were used. After two weeks, the number of live plants was determined and the best substrate was retained for further work.

The second phase consisted in evaluating the effect of the vitroplants origin during weaning in the bins containing only the best substrate previously retained. These bins were then transferred to a greenhouse at a temperature of 28 to 36 °C and a relative humidity of 77 to 85 % for eight weeks. Water was supplied once a day. A total of 180 vitroplants were used at a rate of 30 vitro plants per type (origin of vitroplants) and per elementary plot. The test was conducted in a block design with three repetitions. The number of leaves, roots and the height of each plant were determined.

Breeding plants

For Breeding, the weaned plantlets were transferred into 25 cm x 30 cm polyethylene bags (Fig. 2B). The bags were previously filled with the best weaning substrate (dark soil + sawdust) and perforated to prevent excess water and root asphyxiation. The bags containing the plants were placed under shade. In this second stage of acclimatization, plant care is limited to watering once every two days with tap water for 12 weeks. A monthly fertilization of 2.5 g of potassium sulphate and 1 g of urea per plant was carried out.

The experiment was conducted in randomized complete blocks design consisting of three trials with three repetitions each, where each type of plants corresponds to one test. For each trial, 30 plants were used. Each month, the number of leaves and the number of plants that survived acclimatization were recorded. The survival rate (SR) of acclimatized pineapple plants was calculated according to the following formula:

$$\text{Survival rate (\%)} = \frac{\text{Number of vitroplants acclimated}}{\text{Number of vitroplants released}} \times 100$$



FIGURE 1. Acclimatization of pineapple vitro plants. A: Vitro plants removed from *in vitro* culture media and transferred in bins; B: plantlets transferred in polyethylene bags under shade; C: acclimatized plant, ready to be transferred to the field

Acclimated plants transfer to acclimated plants

Three types of pineapple plants were used in this test:

- Pineapple plants regenerated by direct organogenesis (PM) and acclimated;
- Pineapple plants regenerated by somatic embryogenesis (PE) and acclimated;
- Plants from conventional propagation (PR) obtained from pineapple mother plants, used as controls.

Plants of 30 cm in length were transferred to the field (Fig. 2C). Plants were transplanted onto double line ridges in a three repeat block system. The 5 m × 1 m ridges were previously amended with 1 kg of dolomite and 500 g of calcium triphosphate and then covered with black polyethylene film. The plants were staggered with a spacing of 25 cm on the line and 40 cm between the lines. An elementary plot consisted of 100 plants of each type of plant. The field was regularly maintained and a monthly

fertilization of 2.5 g of potassium sulphate and 1 g of urea per plant has been carried out.

Evaluation of growth and development parameters of acclimated plants transferred to the field

Every two weeks, the height of the plants, the number of leaves emitted per plant, the length and width of leaf D were determined. After ten months of cultivation, the floral induction treatment (TIF) was applied to induce uniform flowering of the plants. Approximately one hundred and fifty days after TIF, the fruits were harvested and the total number of fruits harvested for each type of plant used was determined by counting. The mass of each harvested fruit was determined using a scale (Mettler-Toledo, B154 ®). Fruit weighing greater than or equal to 800 g was considered commercially valuable and the rate was determined according to the following formula:

$$\text{Commercial size fruit rate (FCIC)} = 100 \times \frac{\text{Number of fruits harvested with a weight greater than 800g}}{\text{Number of fruits harvested}}$$

The length of the fruit, the diameter of the fruit and the diameter of the central cylinder (or core) expressed in cm were measured using a caliper. Then, the yield was determined as follow:

$$\text{Yield (Kg/ha)} = \frac{\text{Mass of fruit of commercial size (Kg)}}{\text{Area (ha)}}$$

Statistical analysis

For all experiments performed, STATISTICA 7.1 software was used for statistical analysis. The analysis of variance revealed whether there was a difference between the factors studied. When a difference was observed, the Newman-keuls multiple rank test at 5 % was adopted to separate the averages. The percentage data were processed by the kruskal-wallis test.

RESULTS

Effect of substrates on the survival rate of vitro plants during acclimatization

The results of the various tests carried out made it possible to highlight the behaviour of the vitroplants on the substrates (Table 1). The mixture of dark soil and sawdust was the most favourable to the recovery of vitroplants in an *ex vitro* environment with a survival rate of 87.5 %. Thus, this substrate was used for further tests.

TABLE 1. Survival rate of acclimatized vitroplants on different substrates

Acclimatization substrates	Number of acclimatized plantlets	Plant survival rate (%)
Sea sand	40	17.5±1.82a
Dark soil	40	47.5±3.83b
Dark soil + sawdust	40	87.5±2.17c

±SE: standard error; in the same column, averages followed by the same letter are statically identical at 5% (Newman-keuls test).

Effect of the vitroplants origin on growth and development parameters

During acclimatization, the parameters measured (Table 2) did not change significantly from beginning to the end of

weaning. Plantlets produced by bud culture behaved in the same way as those produced by somatic embryogenesis.

TABLE 2. Evolution of growth parameters of different types of vitroplants during weaning

Parameters	Origin of vitroplants			
	Direct organogenesis		Somatic embryogenesis	
	Before weaning	After weaning	Before weaning	After weaning
Number of leaves	11.34±3.83a	12.72±3.52a	13.00±3.64a	14.04±3.01a
Number of roots	6.53±3.74a	12.52±4.47b	7.94±3.22a	15.22±13.85b
Height (cm)	12.08±0.75a	12.87±0.86a	12.08±0.47a	12.89±0.50a

±SE: standard error; in the same line the averages followed by the same letter are statically identical at 5% (Newman-keuls' test).

Evaluation of survival rates of vitroplants after acclimatization

According to the results reported in Table 3, all the plantlets used from different origins were adapted

identically to the external environment (arab land + sawdust) with 98 % survivals.

TABLE 3 - Survival rate of different types of pineapple vitroplants after acclimatization

Origin of vitroplants	Number of plants acclimated	Plant survival rate (%)
Direct organogenesis	60	98.0±1.63a
Somatic embryogenesis	60	98.3±0.96a

±SE: standard error; The values followed by the same letter are not significantly different at $p = 0.05$; the values represent the average of three repetitions; (Newman-keuls' test)

Impact of plant origin on growth and development parameters in the field

Number of leaves

The results presented in Figure 2 show that in the field the average number of leaves was very significantly

influenced by the origin of the plant. Thus, plants from vitro methods induced the highest number of leaves during the test compared to those from traditional plants (suckers). However, somatic embryogenesis plants produced compared to direct organogenesis plants.

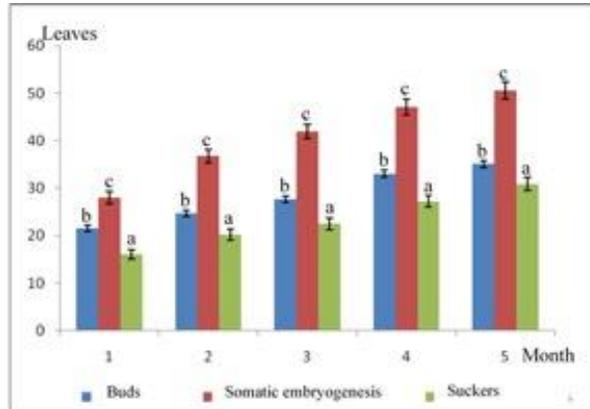


FIGURE 2. Average number of leaves of different types of vitroplants in the field. Histograms with different letters are significantly different at 5% (Newman-keuls' test).

Monthly foliar emission rate

The rate of foliar emission was significantly influenced by the origin of the plants used (Fig. 3). The foliar emission rate of vitroplants was significantly higher than that of traditional plants (suckers). However, the kinetics of emission from somatic embryogenesis was greater than that of plants produced by bud culture. Significant

fluctuations were observed during foliar emission in vitroplants. These fluctuations are characterized by a slowdown phase from the first to the third month and a more accelerated phase from the third month. The rate of foliar emission from traditional plants has been slow during the five months.

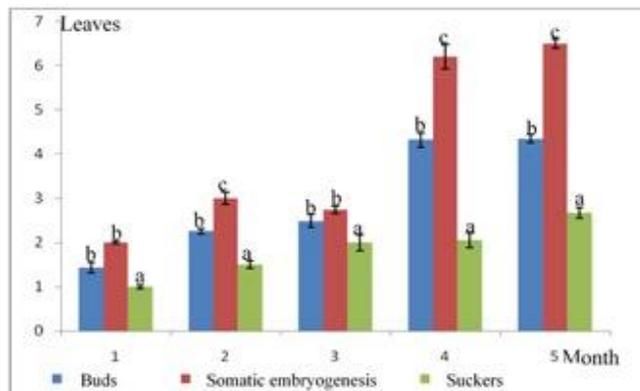


FIGURE 3. The rate of foliar emission of different types of vitroplants in the field. Histograms with different letters are significantly different at 5% (Newman-keuls' test).

Plants height in the field

Field monthly data showed that the height averages of plants produced by bud culture and somatic embryogenesis were significantly different and higher than

those of traditional discards. However, plants obtained from somatic embryogenesis had the highest height means (Fig. 4).

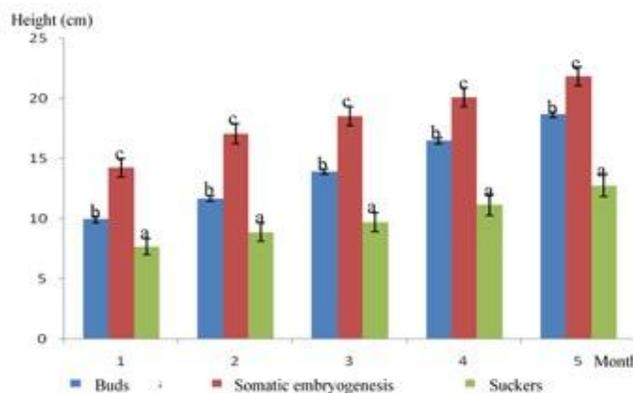


FIGURE 4. Average heights of different types of vitroplants in the field. Histograms with different letters are significantly different at 5% (Newman-keuls' test).

Length of leaf D

The results reported in Table 4 showed that the leaf D length growth was significantly influenced by the origin of the plants. The lengths averages of leaf D of the plants resulting from vitroplants were higher than those of leaf D

of the traditional plants. The results also showed that the length averages of the plants produced with somatic embryogenesis were greater than those of plants produced by bud culture.

TABLE 4 - Leaf D length of the different pineapple plants in the field

Time	Leaf D length (cm)		
	Plants obtained by direct organogenesis	Plants obtained by somatic embryogenesis	Plants obtained by traditional budding
First month	30.48±06.64a	33.10±10.78b	29.50±08.89a
Second month	48.29±03.23b	57.98±08.17c	41.79±11.77a
Third month	69.68±06.24b	88.76±11.99c	59.10±12.21a
Fourth month	88.49±08.16b	100.30±09.55c	73.07±12.50a
Fifth month	97.84±05.07b	117.71±13.40c	82.42±10.32a

±SE: standard error; in the same line the averages followed by the same letter are statically identical at 5% (Newman-keuls' test).

Impact of plants origin on the physical characteristics of fruits

All pineapple plants used in this trial from planting to harvesting (15 months of cultivation), were able to produce fruits (Fig. 5). However, the physical characteristics of the fruits differ according to the origin (Table 6). Thus, vitroplants had better yield (6743 kg/ha and 5979 kg/ha for FE and FM respectively) than plants resulting from traditional budding (4992 kg/ha). The comparison of the average weights of the three types of fruits revealed that FE fruits were significantly heavier

(1124 g) than FM fruits (996 g) and FR fruits (832 g). Similarly, the length and diameter of the three types of fruit evolved in parallel with the weight of the fruit. In addition, 65 % of FE fruits had significantly a higher commercial interest compared to FM fruits (53.70 %) and FR fruits (43 %). However, the results revealed no significant difference between the central cylinders (heart) of the three types of fruit. The results showed that the vitroplants had developed fruits with physical characteristics significantly higher than those of the traditional plants.



FIGURE 5. Fruiting of field-acclimatized pineapple plants A: fruiting of pineapple plants acclimated to the field; B: mature pineapple fruit.

DISCUSSION

The objective of this study was to evaluate vitroplants obtained by direct organogenesis and somatic embryogenesis during acclimatization and in the field. Tests carried out during acclimatization revealed that the nature of the substrate greatly influenced the weaning of vitroplants. Thus, the combination of dark soil and sawdust was the most favourable substrate to vitroplant recovery (87.5 %). This suggests that the combination of substrates would be beneficial for plantlets recovery during acclimatization. Similar results have been reported by several authors (Sidibé *et al.*, 2013; Ayolié *et al.*, 2016) in various plant species (banana, tomato). Indeed, combining sawdust with dark soil would improve the physical properties (structure and texture) of the substrate. That becomes more aerated and less compacting. This allows better water retention and therefore promotes a high survival rate of vitroplants compared to sand and dark soil separately used (El Hamdouni *et al.*, 2000). In addition, the decomposition of sawdust would release mineral elements that enrich the acclimatization substrate. The improvement of these physical and trophic parameters of the substrate, coupled with strict control of environmental conditions (temperature and humidity), had favoured the survival of vitroplants on this substrate.

The sand was the worst substrate to pineapple vitroplants recovery during weaning (17.5 %). This can be explained by the fact that sand offers more heat conditions and retains less water than a fibrous material such as sawdust (Folliot and Marchal, 1990) or lumpy material such as dark soil. On the other hand, the sea sand could have induced salt stress at the origin of certain metabolic disturbances such as reduction of root development, inhibition of hydromineral nutrition, disturbance of photosynthesis and reduction of cell division leading to the death of several plantlets on this substrate (Ould Mohamdi *et al.*, 2011; Achour *et al.*, 2015; Kouadio *et al.*, 2018a). These results are contrary to those reported by Gnamien (2016) in pistachio and Kouadio (2018) in smooth cayenne. Those authors mentioned that sand favoured a better adaptation of vitroplants. However, the combination of sand with any other substrate reduces root aeration. This has a negative influence on vitroplants recovery.

This study also showed that, the origin of vitroplants (bud culture and somatic embryogenesis) did not significantly influence their behaviour during breeding. This suggests that both types of vitroplants are anatomically and physiologically similar. Plants from bud culture would therefore have the same agronomic characteristics as those from somatic embryogenesis. Similar results have been reported by Chatibi *et al.* (1995) in pistachio trees. Indeed, the work of these authors showed that during acclimatization, vitroplants derived from pistachio leaves behave the same way as those derived from buds or cotyledons. while Dibi (2011) demonstrated that in *in vitro* culture, the survival rate of plantlet after acclimatization is generally low. However, our study showed a 98 % pineapple plantlet survival rate after acclimatization. Such high survival rate is rare in *in vitro* culture, although Jain *et al.* (2002) and Le Van *et al.* (2004) have reported similar plantlet results in other plants. Similarly,

Sripaoraya *et al.* (2003) reported plantlet survival rates of 96% during pineapple regeneration via embryogenesis and organogenesis. The growing conditions applied during this experimentation had certainly be beneficial to the development and growth of pineapple plants.

Concerning the evaluation of the behaviour of vitroplants in the natural environment (in planta), the results of this study revealed that pineapple plants, of all origins, survived after their transfer to the field although somaclonal variations were observed by Smith *et al.* (2002). No phenotypic variation in pineapple plants was observed in this study. All plants were able to produce fruit after 15 months. Then, the physical characteristics of the fruit differ according to the origin plants. Thus, compared to the FR fruits produced by pineapple plants from conventional propagation (PR), FM and FE fruits derived from vitro culture (direct organogenesis and somatic embryogenesis) have heavier fruits and higher yields. These results seem to reveal the primordial role of vitro methods in the material savings observed in fruits from vitroplants. This improvement in yield could derived from the physiological potential of the vitroplants because all the plants were cultivated under the same conditions. Indeed, after obtaining the vitroplants by organogenesis (8 weeks), the leaves were used to induce somatic embryogenesis (24 weeks). Rejuvenation being possible in vitro culture (Dibi, 2011), the longer time spent by explants on culture media during somatic embryogenesis would have made it possible to obtain pineapple plants with higher degree of rejuvenation compared to micropropagation. Therefore, the rejuvenation of vitroplants seems to confer more intense physiological potentialities as reported by (Dibi, 2011) in rubber tree. In fact, the juvenility induced by *in vitro* culture has increased the cell division capacity of floral tissues (ovaries, sepals and bracts), stimulated intense synthesis of certain molecules such as sugar and increased fruit size more rapidly. The results revealed an intense differentiation of floral organs in vitroplants compared to traditional plants (suckers). This explain the best yields obtained with vitroplants. Youmbi *et al.* (2005) obtained the same results in banana production where improved banana had better yield compared to traditional variety.

CONCLUSION

Acclimatization is a delicate and important step in *in vitro* culture. Handling in processes will open up new possibilities for farming. This study showed that the combination of dark soil and sawdust allows the best development of pineapple plants for both types of vitroplants considered during weaning.

During breeding, the origin of vitroplants does not influence their behaviour (survival rate, growth and development).

In the field, the study showed that the degree of rejuvenation of plants by *vitro* methods seems to influence vegetative development and the differentiation of floral organs. However, the impact of *in vitro* culture on the biochemical parameters of fruits should be established in order to fully appreciate the influence of the rejuvenation of plants induced by *vitro* culture in pineapple.

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